The triacylglycerol synthesis enzyme DGAT1 also catalyzes the synthesis of diacylglycerols, waxes, and retinyl esters

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Abstract The final step of triacylglycerol biosynthesis is catalyzed by acyl CoA:diacylglycerol acyltransferase (DGAT) enzymes. The two known DGATs, DGAT1 and DGAT2, are encoded by unrelated genes. Although both DGAT1 and DGAT2 knockout mice have reduced tissue triacylglycerol contents, they have disparate phenotypes, prompting us to investigate whether the two enzymes have unrecognized functional differences. We now report that DGAT1 exhibits additional acyltransferase activities in vitro, including those of acyl CoA:monoacylglycerol acyltransferase (MGAT), wax monoester and wax diester synthases, and acyl CoA:retinol acyltransferase (ARAT), which catalyze the synthesis of diacylglycerols, wax esters, and retinyl esters, respectively. These activities were demonstrated in in vitro assays with membranes from insect cells or homogenates from COS7 cells overexpressing DGAT1. Wax synthase and ARAT activities were also demonstrated in intact COS7 cells expressing DGAT1. Additionally, cells and tissues from DGAT1-deficient mice exhibited reduced ARAT activity, and the mice had increased levels of unesterified retinol in their livers on a high-retinol diet. Our findings indicate that DGAT1 can utilize a variety of acyl acceptors as substrates in vitro and suggest that these activities may be relevant to the in vivo functions of DGAT1.—Yen, C-L. E., M. Monetti, B. J. Burri, and R. V. Farese, Jr. **The triacylglycerol synthesis enzyme DGAT1 also catalyzes the synthesis of diacylglycerols, waxes, and retinyl esters. *J. Lipid Res.* 2005.** 46: **1502–1511.**

Supplementary key words acyl CoA:diacylglycerol acyltransferase • retinol (vitamin A) • wax ester • monoacylglycerol

Triacylglycerols (triglycerides) are the primary molecules of energy storage in eukaryotic organisms. In mammals, triacylglycerol biosynthesis is essential for normal physiology and is prominent in the adipose tissue, liver, small intestine, and mammary gland, which all store or secrete triacylglycerols. However, excessive storage of triacylglycerols in human adipose tissue results in obesity, and in nonadipose tissues, it is associated with tissue dysfunction, referred to as lipoxicity (1). Thus, an understanding of the molecular aspects of triacylglycerol biosynthesis is of great importance.

The final step in triacylglycerol biosynthesis, the joining of diacylglycerol and fatty acyl coenzyme A (CoA), is catalyzed by acyl CoA:diacylglycerol *O*-acyltransferase (DGAT). Two DGAT enzymes have been identified. DGAT1, a member of a gene family that includes acyl CoA:cholesterol acyltransferase (ACAT) 1 and ACAT2 (2–4), is a protein of ~500 amino acids in most species (5, 6). It is very hydrophobic, has multiple predicted membrane-spanning domains (5), and like ACAT1 (7), forms homodimers and homotetramers (8). DGAT1 is expressed in most tissues, with the highest expression levels in the small intestine, testis, adipose tissue, mammary gland, and skin (5). DGAT2, on the other hand, is a member of a larger gene family whose members include acyl CoA:monoacylglycerol acyltransferase (MGAT) 1, MGAT2, MGAT3, and wax synthases (9–16). DGAT2 enzymes of different species have ~400 amino acids, are less hydrophobic than DGAT1, and have only one or two predicted membrane-spanning domains (10). DGAT2 is expressed highly in tissues that figure prominently in triacylglycerol metabolism, including liver and adipose tissues (10). Despite their similar enzymatic properties in in vitro assays, DGAT1 and DGAT2 share little, if any, sequence similarity (5, 10).

The in vivo functions of DGAT1 and DGAT2 have been analyzed in gene-targeted mice deficient in either enzyme (17, 18). DGAT1-deficient (*Dgat1*$^{-/-}$) mice are viable, have modest reductions in tissue triacylglycerol content, and have normal plasma triacylglycerol levels (17). These mice are protected from diet-induced and some genetic forms of obesity through an increase in energy expenditure (17,

Manuscript received 31 January 2005 and in revised form 7 April 2005.

Published, JLR Papers in Press, April 16, 2005.
DOI 10.1194/jlr.M500036-JLR200

Abbreviations: ACAT, acyl CoA:cholesterol acyltransferase; ARAT, acyl CoA:retinol acyltransferase; CoA, coenzyme A; DGAT, acyl CoA:diacylglycerol acyltransferase; LRAT, lecithin:retinol acyltransferase; MEF, mouse embryonic fibroblast; MGAT, acyl CoA:monoacylglycerol acyltransferase; TLC, thin-layer chromatography.

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19). They exhibit increased sensitivity to insulin and leptin and are protected from diet-induced insulin resistance (19, 20). These phenotypic effects appear to be due in part to altered endocrine function of the white adipose tissue (21). Unexpectedly, *Dgat1*$^{-/-}$ mice also have abnormalities in tissue-specific developmental processes. Their skin is characterized by atrophy of the sebaceous glands and hair loss, which is most prominent in postpubertal male mice. Their fur lacks wax diesters (22), which are the major component of skin surface lipids in mice. Female *Dgat1*$^{-/-}$ mice exhibit a lactation defect and do not secret milk due to impaired development of the mammary epithelium (17, 23). This aspect of the phenotype appears to reflect the absence of DGAT1 both in the mammary epithelium and in the surrounding stromal tissue (23).

The absence of DGAT2 has a more profound effect on triacylglycerol metabolism. DGAT2-deficient (*Dgat2*$^{-/-}$) mice have intrauterine growth retardation and die within hours after birth (18). They have severely reduced triacylglycerols and lack substrates for energy metabolism. They also lack essential fatty acids and specific skin lipids that are crucial for the function of the epidermal water barrier (18). In yeast, the DGAT2 homolog Dga1p accounts for the majority of triacylglycerol synthesis (24), whereas the DGAT1 homologs Are1p and Are2p account for only a minor portion (25).

The disparate phenotypes of DGAT1- and DGAT2-deficient mice prompted us to investigate whether the two enzymes have previously unrecognized differences in their biochemical functions. In this study, we sought to determine whether DGAT1 and DGAT2 have other substrates and, more specifically, whether either DGAT can use additional fatty acyl acceptors as substrates.

MATERIALS AND METHODS

Expression studies in insect cells

Mouse DGAT1 (accession number AF078752) and DGAT2 (accession number AF384160) were tagged with an N-terminal FLAG epitope (MGDYKDDDDG, epitope underlined) and expressed in *Spodoptera frugiperda* (Sf9) insect cells by baculovirus infections as described (5, 10–12). Wild-type baculovirus infections were used as a negative control. Expression of DGAT1 and DGAT2 proteins was demonstrated by immunoblotting of membrane proteins (5 μg) with an anti-FLAG M2 antibody (Sigma-Aldrich Co.; St. Louis, MO).

In vitro acyltransferase assays

For initial screening of acyl aceptors, acyltransferase activities were assayed with 25 μM acyl donor and 100 μM acyl acceptor as described (11). Nonpolar acyl acceptors (diacylglycerol, monoacylglycerol, 1-hexadecanol, 1,2-hexadecandiol, cholesterol, and retinol) were dissolved in acetone, which accounted for 5% of 200 μl reactions. Retinol was dissolved in dimethylsulfoxide in some experiments, and results were identical to those obtained with retinol dissolved in acetone. Reactions were started by adding membranes (from insect cells) or total protein homogenates (from mammalian cells or mouse tissues) and stopped after 10 min (increases in reaction products were linear for up to 30 min) by adding 4 ml of chloroform-methanol (2:1; v/v). The extracted lipids were dried, separated by thin-layer chromatography (TLC) with hexane-ethyl ether-acetic acid (80:20:1; v/v/v), visualized with iodine vapor, and identified by comparison with lipid standards. For experiments with radiolabeled substrates, TLC plates were scanned with an imaging scanner (Bioscan AR-2000; Washington, D.C.) or exposed to X-ray film, and bands were scraped to assess the incorporation of radioactivity into lipid products. All assays were performed in duplicate, and experiments were repeated two to three times.

MGAT (EC 2.3.1.22), DGAT (EC 2.3.1.20), acyl CoA:long-chain fatty alcohol *O*-acyltransferase (wax synthase; EC 2.3.1.75), and acyl CoA:retinol *O*-acyltransferase (ARAT; EC 2.3.1.76) activities were detected by incorporation of [^{14}C]palmitoyl CoA (25 μM; specific activity, ~20,000 cpm/nmol; Amersham Biosciences, Piscataway, NJ) into diacylglycerol, triacylglycerol, wax monoester and wax diester, and retinyl ester in the presence of added *sn*-2 monooleoylglycerol, *sn*-1,2 dioleoylglycerol, 1-hexadecanol, 1,2-hexadecandiol, and all-*trans* retinol, respectively. Stereoisomers and derivatives of retinol (all-*trans* and 13-*cis* retinol, all-*trans* retinal, and all-*trans* retinoic acids) were from Sigma-Aldrich. The dependence of MGAT, wax synthase, and ARAT activities on acyl acceptors as substrates was determined with assays using various concentrations of acyl acceptor (*sn*-2 monooleoylglycerol, 1-hexadecanol, 1,2-hexadecandiol, and all-*trans* retinol, respectively) and 50 μM [^{14}C]palmitoyl CoA (specific activity, ~20,000 cpm/nmol). The acyl acceptors were serially diluted beforehand to maintain the percentage of acetone in each reaction at 5%. Likewise, the dependence of MGAT, wax monoester synthase, and ARAT activity on acyl donor as substrates was determined with assays using various concentrations of palmitoyl CoA and the respective radiolabeled acyl acceptor (200 μM [^{3}H]*sn*-2 monooleoylglycerol, specific activity ~3,000 cpm/nmol; 100 μM [^{14}C]1-hexadecanol, specific activity ~6,000 cpm/nmol; 50 μM [^{3}H]all-*trans* retinol, specific activity ~330,000 cpm/nmol; American Radiolabeled Chemicals, St. Louis, MO).

Expression studies in mammalian cells

The FLAG-tagged mouse DGAT1 cDNA was subcloned into a pcDNA3 expression vector and transfected into COS-7 cells with Fugene 6 (Roche Diagnostics; Chicago, IL). The human DGAT1 coding sequence (accession number NM_012079) was amplified from human liver cDNA (Clontech; Palo Alto, CA) by PCR (Takara Ex Taq; Panvera, Madison, WI) and was subcloned without the FLAG tag into pIRESneo2 vector (Clontech). β-Galactosidase, FLAG-tagged mouse ACAT2 (accession number AF078751), DGAT2, MGAT1 (accession number AF078752), and human MGAT2, (accession number AY157608) were expressed as controls. Cells were harvested 48 h after transfection. Expression of FLAG-tagged proteins was analyzed by immunoblotting of homogenates (20 μg of protein) with the anti-FLAG antibody. The in vitro acyltransferase activities of total homogenates were assayed with labeled palmitoyl CoA as described above for proteins expressed in insect cells.

Wax and retinol esterification in cultured cells

The incorporation of [^{14}C]1-hexadecanol into wax monoester and of [^{3}H]retinol into retinyl esters in cultured cells was measured in COS-7 cells overexpressing DGAT1. COS-7 cells were plated in six-well dishes (10^5/well) for 24 h and transfected with vectors containing cDNAs for DGAT1 or control enzymes; 24 h later, cells were incubated in medium containing 5 μM [^{14}C]1-hexadecanol (specific activity, ~5,500 cpm/nmol) or 2.5 μM [^{3}H]retinol (specific activity, ~200,000 cpm/nmol) for 18 h. Neutral lipids were extracted, separated by TLC, visualized with a Bioscan imaging scanner, and scraped to assess the incorporation of radioactivity into wax and retinyl esters.

In vitro assay and labeling studies with mouse embryonic fibroblasts

Mouse embryonic fibroblasts (MEFs) were established from DGAT1-deficient embryos and their wild-type littermates as described (10). The in vitro acyltransferase activities of total homogenates from primary MEFs (passage 3) were assayed with labeled palmitoyl CoA as described above for insect proteins. For labeling of intact cells, primary MEFs were plated in triplicate (4×10^5/100 mm dish) and, 24 h later, incubated with medium containing 5 μM [^{3}H]retinol (specific activity, ~70,000 cpm/nmol) for 18 h (similar results, with proportionally less incorporation of radioactivity, were observed after 6 h of incubation). Neutral lipids were analyzed as described above.

Measurements of retinol and retinyl esters

Retinoid levels were measured with reverse-phase HPLC methods. For MEFs, cellular retinol levels were measured in three samples of 3–10 $\times$ 10^6 cells from each cell line. Cells were cultured in media containing 10% fetal bovine serum that contained 0.5 μM retinol and 0.25 μM retinyl esters. For mouse studies, male wild-type and *Dgat1*$^{-/-}$ mice (19 weeks old; >96% C57BL/6J genetic background) were maintained on a regular chow diet (containing 10 kcal% fat, PicoLab 5053; Purina Mill, Brentwood, MO) and then either kept on chow or switched to a high-fat diet (containing 45 kcal% fat, TD88137; Harlan, Madison, WI) for 1 week before tissues were harvested. The chow diet contained ~10 IU/g of vitamin A (mainly retinyl acetate), and the high-fat diet contained ~20 IU/g of vitamin A (mainly retinyl palmitate).

Retinoid levels in MEFs and mouse livers were measured in the laboratory of B. J. Burri (USDA, Davis, CA) (26) and levels in diets and serum were measured by Craft Technologies (Wilson, NC) (27). In one experiment, 12-week-old male wild-type and *Dgat1*$^{-/-}$ mice were maintained on a high-vitamin A diet (containing 160 IU/g of retinyl palmitate and 10 kcal% fat, D99102101; Research Diets, New Brunswick, NJ) for 3 weeks, and tissue retinoid levels were measured by Craft Technologies.

RESULTS

DGAT1 expressed in insect cells possesses multiple acyltransferase activities

To examine the acyltransferase activities of DGAT1 and DGAT2, FLAG-tagged versions of their murine cDNAs were expressed in insect cells. These proteins migrated on SDS-PAGE at the expected molecular masses, and the levels of expression were comparable, as demonstrated by immunoblotting with an anti-FLAG antibody (**Fig. 1A**). In control experiments, membranes expressing either DGAT incorporated more [^{14}C]palmitoyl CoA into triacylglycerols than did membranes from cells infected with wild-type baculovirus (Fig. 1B), verifying that both expressed enzymes were active. As reported previously (11, 12), both DGAT1 and DGAT2 also possess in vitro MGAT activity, incorporating [^{14}C]palmitoyl CoA into diacylglycerol and triacylglycerols in the presence of monoacylglycerol (Fig. 1B).

We next explored whether DGAT1 or DGAT2 could utilize other acyl acceptors as substrates in vitro. With [^{14}C] palmitoyl CoA as the acyl donor, membranes expressing DGAT1, but not those expressing DGAT2 or control viral

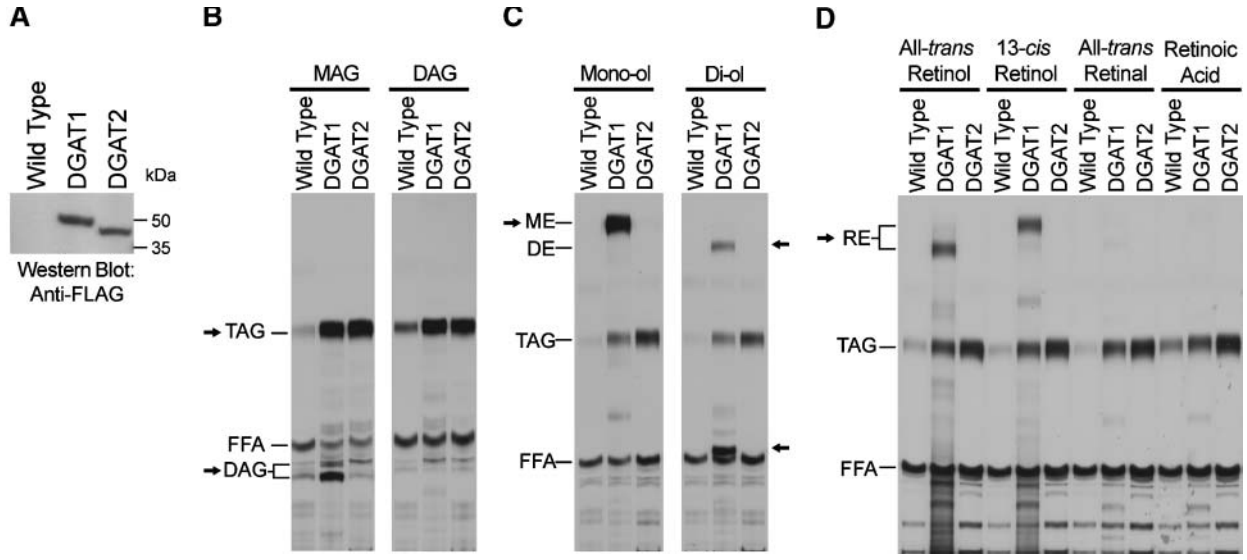

Fig. 1. Expression of acyl CoA:diacylglycerol acyltransferase 1 (DGAT1) and DGAT2 in Sf9 insect cells. A: Immunoblots of insect cell membranes. Expression of DGAT1 and DGAT2 was demonstrated by immunoblotting with an anti-FLAG antibody. Membrane proteins (5 μg) from Sf9 cells infected with wild-type virus, FLAG-tagged DGAT1 (DGAT1), or FLAG-tagged-DGAT2 (DGAT2) recombinant baculoviruses were analyzed. B–D: Acyltransferase activities conferred by expression of DGAT1. Acyl CoA:monoacylglycerol acyltransferase (MGAT) and DGAT (B), wax monoester and diester synthase (C), and acyl CoA:retinol acyltransferase (ARAT) (D) activities were detected by incorporation of [^{14}C]palmitoyl coenzyme A (CoA) into diacylglycerol (DAG), triacylglycerol (TAG), wax monoester (ME), wax diester (DE), and retinyl ester (RE) in the presence of 100 μM added *sn*-2 monooleoylglycerol (MAG), *sn*-1,2 dioleoylglycerol (DAG, top label), 1-hexadecanol (Mono-ol), 1,2-hexadecandiol (Di-ol), and retinol (all-*trans* and 13-*cis* retinol), respectively. FFA, free fatty acid. The lipid products were extracted, dried, separated by thin-layer chromatography, and detected by exposure to X-ray film as described. Arrows indicate the respective product(s) of each acyltransferase activity. In (C), the prominent acylation product when 1,2-hexadecandiol was provided is most likely the intermediate 2-hydroxylhecadecyl hexadecanoate (lower arrow). Chromatographs shown are representative of three independent experiments.

proteins, catalyzed the incorporation of the acyl group into wax monoesters, wax diesters, and retinyl esters in the presence of 1-hexadecanol, 1,2-hexadecandiol, and retinol, respectively (Fig. 1C, D). Both all-*trans* retinol and 13-*cis* retinol served as substrates for the DGAT1-mediated retinol acyltransferase activity, but all-*trans* retinal and retinoic acid did not, consistent with a requirement for a free hydroxyl group on the end of the retinoid side chain (Fig. 1D). Cholesterol, glycerol, glycerol-3-phosphate, and 1-acylglycerol-3-phosphate did not serve as substrates (not shown).

To examine whether the acyltransferase activities of DGAT1 depend on the presence of appropriate substrates, we first varied the acyl acceptor concentrations in assays containing 50 μM [^{14}C]palmitoyl CoA. In general, enzymatic activity increased with the concentration of each acyl acceptor (**Fig. 2A–D**). Although these experiments were not designed to calculate apparent K_m and V_{max}, under the assay condition used, we found that the maximal enzymatic activity measured was highest with monoacylglycerol as the acceptor, a condition that reflects both MGAT and DGAT activities (Fig. 2A). The apparent maximal activities observed with other acceptors were ~10% of that for monoacylglycerol but were still considerable (between 0.5 and 1.5 nmol/min/mg protein) (Fig. 2A–D).

We also examined these acyltransferase activities at different concentrations of palmitoyl CoA and a fixed concentration of radiolabeled acyl acceptor as tracers (200 μM [^{3}H] *sn*-2-monooleoylglycerol for MGAT activity, 100 μM [^{14}C]1-hexadecanol for wax synthase activity, and 50 μM [^{3}H] all-*trans* retinol for ARAT activity). In each case, the acyltransferase activity increased with increasing acyl CoA concentrations (**Fig. 3A–C**). Again, the activity was highest with *sn*-2-monoacylglycerol as the acyl acceptor, and the levels of this MGAT activity did not reach plateau even when the concentration of palmitoyl CoA was as high as 200 μM (Fig. 3A–C). Because these assays utilized radiolabeled acyl acceptors as substrates, they provide strong evidence that DGAT1 possesses in vitro MGAT, wax synthase, and ARAT activities.

DGAT1 possesses wax synthase and ARAT activities in mammalian cells

To determine whether DGAT1 possesses wax synthase and ARAT activities in mammalian cells, we transiently overexpressed mouse DGAT1 and control enzymes in COS-7 cells and assessed their enzymatic activities in cellular homogenates. The expression of FLAG-tagged DGAT and control proteins was demonstrated by immunoblotting (**Fig. 4A**). In control assays with diacylglycerol and [^{14}C]palmitoyl CoA as the tracer, homogenates of COS-7 cells overexpressing DGAT1 or DGAT2 incorporated more radioactivity into triacylglycerols than did controls overexpressing other enzymes, including β-galactosidase (LacZ), ACAT2, MGAT1, and MGAT2 (Fig. 4B). Homogenates of cells overexpressing ACAT2 incorporated more [^{14}C]palmitoyl CoA into cholesterol esters than did the other enzymes. With 1-hexadecanol as the acyl acceptor, only homogenates from cells expressing DGAT1 exhibited wax synthase activity (Fig. 4C). Similarly, with all-*trans* retinol as

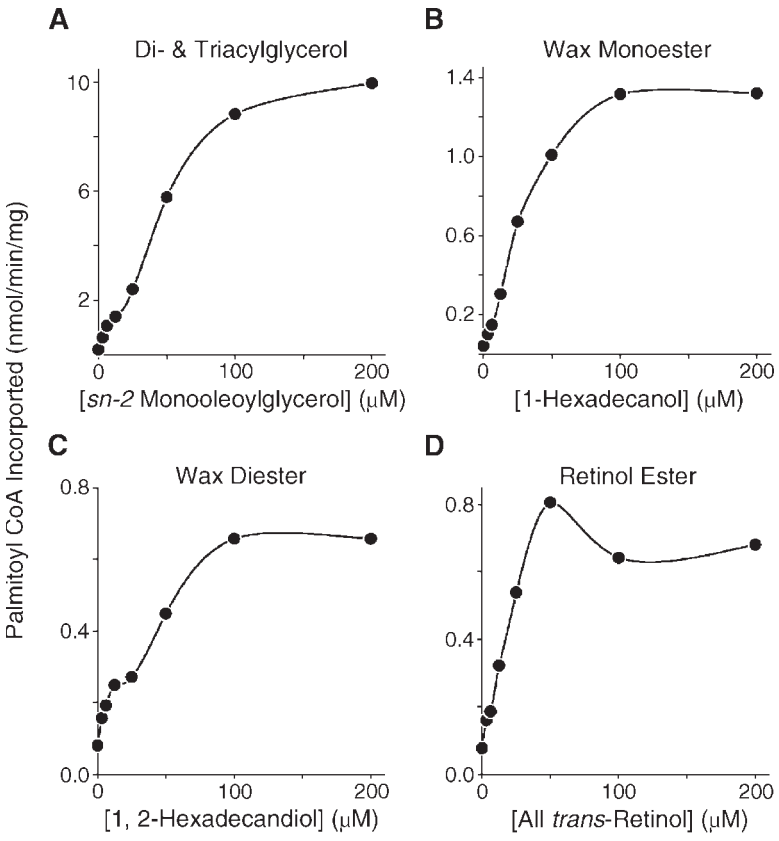

Fig. 2. Dependency of acyltransferase activities in membrane expressing DGAT1 on acyl acceptor concentrations. A: Acylglycerol acyltransferase (both DGAT and MGAT) activities assessed by the amount of [^{14}C]palmitoyl CoA incorporated into both diacylglycerol and triacylglycerol in the presence of different concentrations of *sn*-2 monooleoylglycerol. B: Wax monoester synthase activity assessed by the amount of [^{14}C]palmitoyl CoA incorporated into wax monoester in the presence of different concentrations of 1-hexadecanol. C: Wax diester synthase activity assessed by the amount of [^{14}C]palmitoyl CoA incorporated into wax diester in the presence of different concentrations of 1,2-hexadecandiol. D: ARAT activity assessed by the amount of [^{14}C]palmitoyl CoA incorporated into retinyl ester in the presence of different concentrations of all-*trans* retinol. Values are the mean of duplicate measurements and are representative of two experiments.

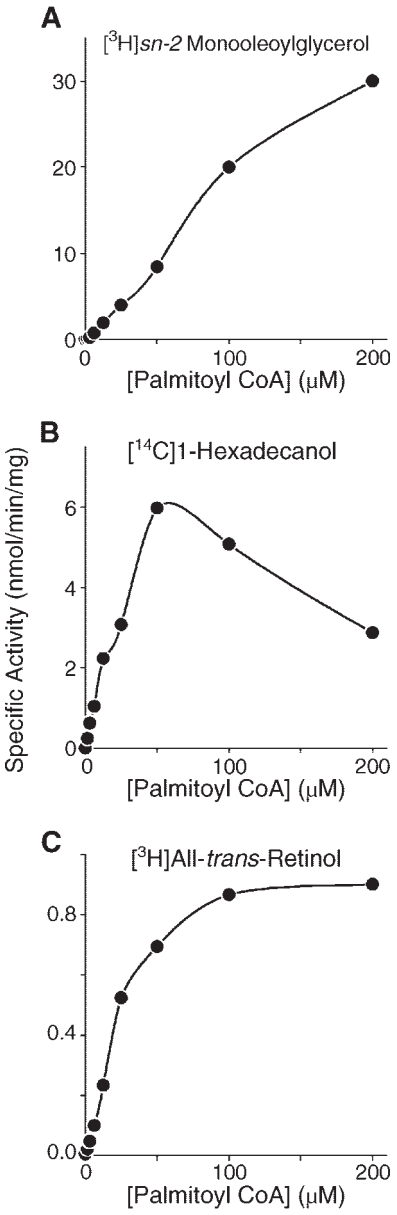

Fig. 3. Dependency of acyltransferase activities in membrane expressing DGAT1 on acyl donor concentrations. A: MGAT activity assessed by the amount of [^{3}H]*sn*-2 monooleoylglycerol incorporated into both diacylglycerol and triacylglycerol in the presence of different concentrations of palmitoyl CoA. B: Wax monoester synthase activity assessed by the amount of [^{14}C]1-hexadecanol incorporated into wax monoester in the presence of different concentrations of palmitoyl CoA. C: ARAT activity assessed by the amount of [^{3}H]all-*trans* retinol incorporated into retinyl ester in the presence of different concentrations of palmitoyl CoA. Values are the mean of duplicate measurements and are representative of two experiments.

the acceptor, homogenates from cells expressing DGAT1 exhibited ARAT activity (Fig. 4D). In some experiments, homogenates from cells expressing MGAT1 also exhibited very low levels of ARAT activity.

To determine whether DGAT1 exhibits wax synthase and ARAT activities in intact cells, we measured the accumulation of radiolabeled substrates ([^{14}C]1-hexadecanol or [^{3}H]all-*trans* retinol) in esterification products in COS-7 cells overexpressing mouse DGAT1 and control enzymes (**Fig. 5A**). (We did not examine MGAT activity in intact cells overexpressing DGAT1 because monoacylglycerol, the MGAT reaction substrate, can be hydrolyzed before uptake by cells, and because diacylglycerol, the MGAT reaction product, can be converted to other lipids and does not accumulate in significant amounts.) Wax ester synthesis was highest in cells overexpressing DGAT1 (~10-fold of LacZ controls; Fig. 5B). Cells expressing DGAT2, and to a lesser extent cells overexpressing MGAT1, also synthesized more wax esters than did control cells. Retinol esterification levels were highest in cells overexpressing DGAT1 (~5-fold of LacZ controls; Fig. 5C). Cells overexpressing MGAT1 also exhibited higher levels of retinol esterfication than did control cells.

In addition, we examined COS-7 cells expressing human DGAT1 without the FLAG tag epitope. Like mouse DGAT1, human DGAT1 exhibited wax synthase and ARAT activities both in in vitro assays and in intact cell esterification assays (not shown). These findings also showed that the presence of the FLAG tag at the N-terminus of DGAT1 did not affect these additional acyltransferase activities.

Cells and tissues lacking DGAT1 exhibit reduced ARAT activity

Because the genes encoding ARAT enzymes have not been identified, we further characterized the ARAT activity of DGAT1 by examining whether ARAT activity was diminished in mouse cells and tissues lacking DGAT1. We first examined whether primary MEFs from *Dgat1*$^{-/-}$ mice have decreased in vitro ARAT activity. In a control experiment with [^{14}C]palmitoyl CoA as the acyl donor and diacylglycerol as the acceptor, homogenates from two independent lines of *Dgat1*$^{-/-}$ MEFs exhibited less triacylglycerol synthesis than did homogenates from wild-type MEFs, as expected (not shown). With [^{14}C]palmitoyl CoA as the acyl donor and all-*trans*-retinol as the acyl acceptor, ARAT activity was detectable in homogenates of three different wild-type MEF cell lines (**Fig. 6A**). In contrast, ARAT activity was nearly absent in homogenates of two different lines of *Dgat1*$^{-/-}$ MEFs.

We next examined retinol esterification in intact wild-type and *Dgat1*$^{-/-}$ MEFs by measuring the accumulation of radioactivity in retinyl esters after 18 h incubation with [^{3}H]all-*trans*-retinol. Levels of radiolabeled retinyl esters in two different cell lines of *Dgat1*$^{-/-}$ MEFs were less than one-half of those in wild-type MEF lines (Fig. 6B). We excluded a dilution effect of the tracer as a cause of the reduced retinol esterification in *Dgat1*$^{-/-}$ MEFs by measuring the retinol content in wild-type and *Dgat1*$^{-/-}$ MEFs. We found that the retinol levels were similar in wild-type and *Dgat1*$^{-/-}$ MEFs [1.68 ± 1.25 vs. 1.45 ± 0.004 pmol/10^6 cell (mean ± SD)] and that the basal cellular retinol levels were 3 orders of magnitude lower than the levels of labeled retinol taken up by cells (0.74–3.06 pmol/10^6 cell vs. 2–3 nmol/10^6 cell). The residual retinol esterification in *Dgat1*$^{-/-}$ MEFs is presumably due to a nonARAT activity, possibly that of lecithin:retinol acyltransferase (LRAT),

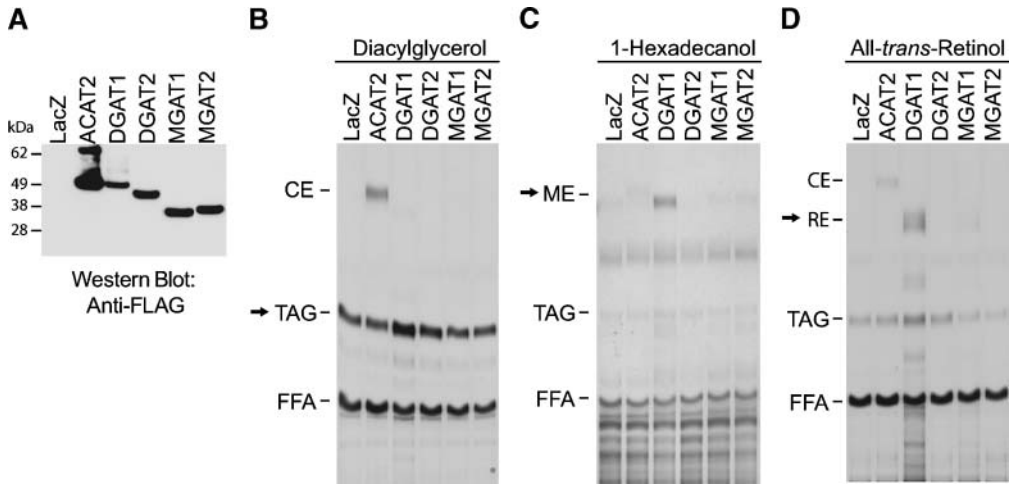

Fig. 4. Expression of DGAT1 in mammalian cells confers wax synthase and ARAT activity in in vitro assays. A: Immunoblotting of FLAG-tagged proteins demonstrating expression of FLAG-tagged DGAT1 and control proteins. Cells were transfected with expression vectors containing cDNAs for β-galactosidase (LacZ) or FLAG-tagged versions of acyl CoA:cholesterol acyltransferase 2 (ACAT2), DGAT1, DGAT2, MGAT1, or MGAT2. B–D: DGAT (B), wax synthase (C), and ARAT (D) activities in COS-7 cell homogenates conferred by expression of DGAT1. [^{14}C]palmitoyl CoA was used for enzyme assays. Arrows indicate the incorporation of [^{14}C]palmitoyl CoA into triacylglycerol (TAG), wax monoester (ME), and retinyl ester (RE). CE, cholesterol ester. FFA, free fatty acid. Chromatographs shown are representative of three independent experiments.

which uses lecithin (phosphatidylcholine) instead of fatty acyl CoA as the acyl donor.

We next examined in vitro ARAT activities in liver, testis, and kidney homogenates from wild-type and *Dgat1*$^{-/-}$ mice. In control assays, ACAT activities were similar in homogenates from wild-type and *Dgat1*$^{-/-}$ tissues (**Fig. 7A**). In *Dgat1*$^{-/-}$ tissue homogenates, DGAT activity was reduced by more than 80% (Fig. 7B) and ARAT activity was reduced by 75–90% (Fig. 7C).

Finally, we assessed whether retinol metabolism is perturbed in *Dgat1*$^{-/-}$ mice. Mice were fed diets containing different levels of retinol, and retinol and retinyl esters were measured in the liver, where the majority of retinol is stored. In mice fed standard chow (~10 IU/g retinol), the hepatic content of unesterified retinol was similar in wild-type and *Dgat1*$^{-/-}$ mice (**Fig. 8A**). However, in mice fed either a high-fat diet (~20 IU/g retinol) or a high-retinol diet (160 IU/g retinol), unesterified retinol was 2- to 3-fold more abundant in *Dgat1*$^{-/-}$ livers (Fig. 8A). Interestingly, the hepatic content of retinyl esters was not reduced in *Dgat1*$^{-/-}$ livers from mice fed any of the diets, and in fact, retinyl ester levels were significantly higher in livers of *Dgat1*$^{-/-}$ mice fed the high-fat diet (Fig. 8B).

DISCUSSION

In this study, we show that mammalian DGAT1 possesses MGAT, wax synthase, and ARAT activities as well as DGAT activity in in vitro assays of membranes of insect cells or homogenates of mammalian cells overexpressing DGAT1. Wax synthase and ARAT activities catalyzed by DGAT1 were demonstrated in intact cells, and ARAT activity was reduced in DGAT1-deficient cells and tissues. These additional activities were not found for DGAT2, with the exception of increased wax synthesis in intact cells overexpressing DGAT2, which was reported previously (15). Thus, unlike DGAT2, which appears to function primarily as a DGAT, DGAT1 possesses multiple acyltransferase activities.

These results are consistent with the evolutionary history of the two enzymes. The DGAT2 homolog in *Saccharomyces cerevisiae*, Dga1p, plays a major role in triacylglycerol synthesis (24, 28), whereas the DGAT1 homologs, Are1p and Are2p, play minor roles (24). Instead, Are1p and Are2p are involved in sterol esterification (29). Because the relatives of DGAT1 in lower organisms were not primarily involved in esterifying diacylglycerol, DGAT1 must have acquired the ability to utilize diacylglycerol as a substrate in higher organisms during evolution. Therefore, it is plausible that the substrate recognition site of DGAT1 is less discriminating than the corresponding site of DGAT2. Moreover, DGAT1 may have also acquired the ability to utilize other fatty acyl acceptors, such as monoacylglycerol, long-chain alcohols, and retinol.

The demonstration that DGAT1 possesses MGAT activity, as noted briefly in reports of the cloning of MGAT genes (11, 12), suggests that DGAT1 can catalyze the sequential esterification of two fatty acyl moieties to convert monoacylglycerol to diacylglycerol and then to triacylglycerol. This ability is consistent with a role for DGAT1 in the reesterification of monoacylglycerol and diacylglycerol generated by triacylglycerol hydrolysis in cells. Whether this is a function of DGAT1 in intact cells requires further testing.

DGAT1 catalyzed the synthesis of both wax monoesters and wax diesters. The combination of wax monoester synthase and DGAT activities is similar to that of an unrelated

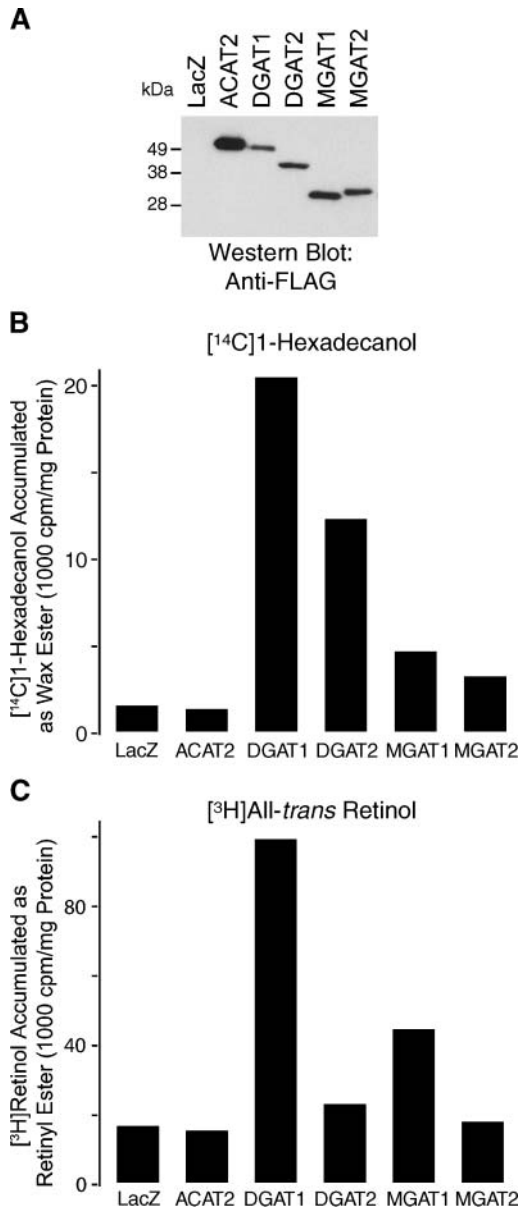

Fig. 5. Expression of DGAT1 in mammalian cells increases esterification of fatty acyl alcohol and retinol in intact cells. A: Immunoblotting of FLAG-tagged proteins demonstrating expression of FLAG-tagged DGAT1 and control proteins. B and C: Increased incorporation of fatty acyl alcohol into wax esters and of retinol into retinyl esters in COS-7 cells overexpressing DGAT1. Cells were plated in six-well plates, transfected with DGAT1 or control proteins, and 24 h later, incubated in medium containing 5 μM [^{14}C]1-hexadecanol (specific activity, ~5,500 cpm/nmol) or 2.5 μM [^{3}H]retinol (specific activity, ~200,000 cpm/nmol) for 18 h. Neutral lipids were separated by TLC, visualized with a Bioscan imaging scanner, and scraped to assess the incorporation of radioactivity. Values represent results from six pooled six-well dishes of each group. Similar results were observed in three independent experiments.

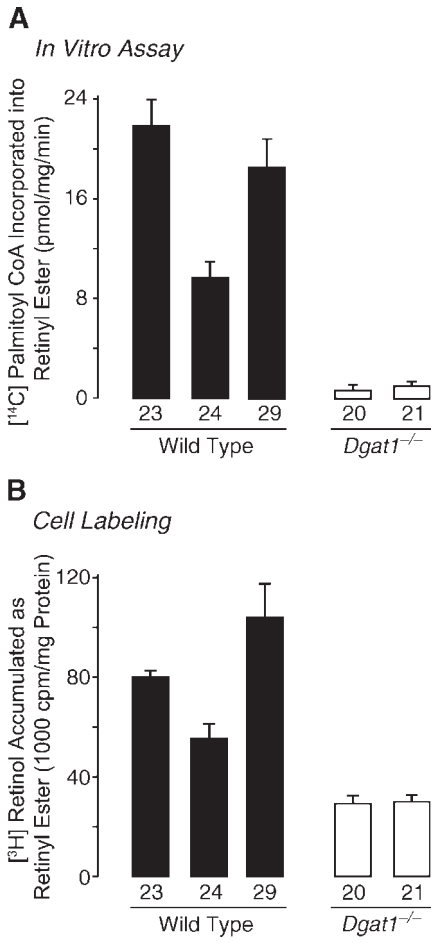

Fig. 6. Mouse embryonic fibroblasts (MEFs) lacking DGAT1 exhibit reduced ARAT activity. A: Reduced in vitro ARAT activities in MEF homogenates lacking DGAT1. Primary MEFs were established from DGAT1-deficient embryos and their wild-type littermates as described (10). Each number represents an independent line of MEF cells. [^{14}C]palmitoyl CoA was used for enzyme assays. Values are mean ± SD of four measurements. B: Decreased incorporation of retinol into retinyl esters in MEFs lacking DGAT1. Cells were plated in triplicate and, 24 h later, incubated in medium containing 5 μM [^{3}H]retinol (specific activity, ~70,000 cpm/nmol) for an additional 18 h. Values are mean ± SD of measurements from three plates of each clone and are representative of two repeated experiments.

wax synthase/DGAT found in bacteria (30). It is not known whether, like DGAT1, the bacterial bifunctional enzyme is active in forming wax diesters. The wax monoester synthase activity of DGAT1 agrees with a recent report showing that DGAT1 overexpression in intact cells increases wax synthesis (15). The wax diester synthase activity of DGAT1 may help to explain the deficiency of type II wax diesters in the fur lipids of *Dgat1*$^{-/-}$ mice (22). However, because wax diesters are present in the fur of leptin-deficient *Dgat1*$^{-/-}$ mice (22), other wax synthases must exist. Our results show that DGAT2 overexpression also increased wax monoester synthase activity in intact cells, a finding that was recently reported (15), although we did not detect either wax monoester or diester synthase activity in in vitro assays of DGAT2. Additional wax synthases (members of the DGAT2 gene family) that catalyze the synthesis of wax monoesters (15, 16), but not wax diesters, were recently identified (C-L. Yen et al., unpublished observations).

Although ARAT activity was reported decades ago (31–34), the ARAT genes have not been identified. The cur-

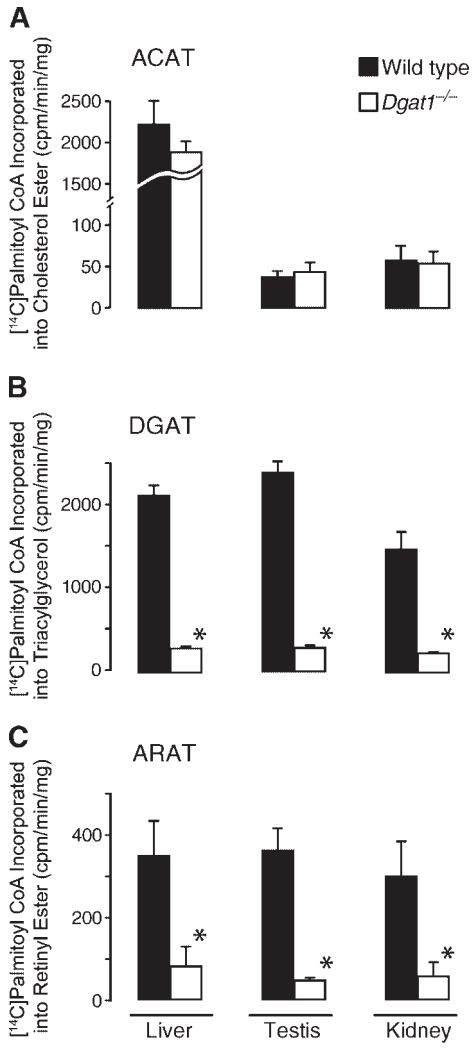

Fig. 7. Reduced in vitro ARAT activities in liver, testis, and kidney of DGAT1-deficient mice. Tissue homogenates were from wild-type and *Dgat1*$^{-/-}$ mice fed a regular chow. The activities of ACAT (A), DGAT (B), and ARAT (C) were assessed by the incorporation of [^{14}C] palmitoyl CoA (25 μM) into cholesterol esters, retinyl ester, and triacylglycerol, respectively. Values are mean ± SD of measurements from four mice in each group. * $P < 0.001$ versus wild-type mice (two-way ANOVA followed by Tukey test for multiple comparisons).

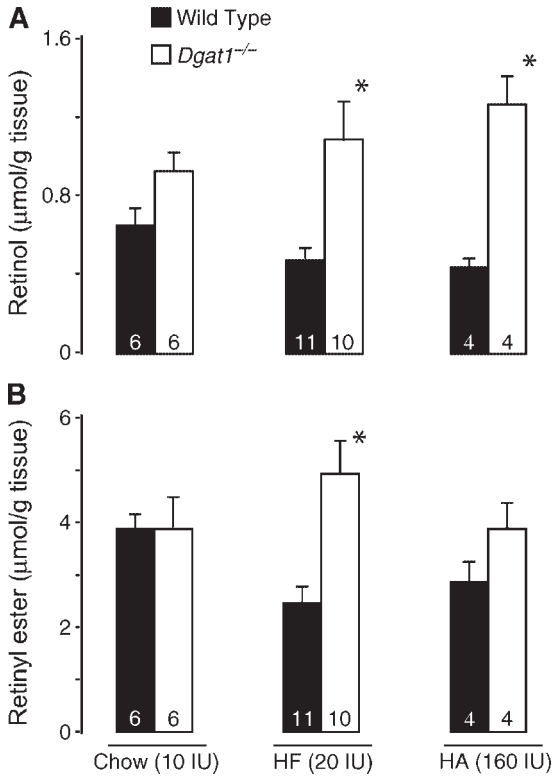

Fig. 8. Retinol and retinyl ester content in the livers of DGAT1-deficient mice. Wild-type and *Dgat1*$^{-/-}$ mice were fed a regular chow (containing 10 IU/g retinol), a high-fat diet (HF, 20 IU/g retinol), or a high-vitamin A diet (HA, 160 IU/g retinol). Levels of retinol (A) and retinyl esters (B) were measured by HPLC methods. Values are mean ± SEM. (The number of mice is indicated in the bars.) * $P < 0.05$ versus wild-type mice (two-way ANOVA followed by Tukey test for multiple comparisons).

rent findings suggest that DGAT1, which is expressed in tissues reported to have ARAT activity, contributes to ARAT activity in vivo. DGAT1 exhibited ARAT activity in in vitro assays and accounted for a large portion of ARAT activity in the liver, testis, and kidney of mice. DGAT1 deficiency also perturbed retinol metabolism in the livers of *Dgat1*$^{-/-}$ mice. The physiological significance of ARAT, however, is not clear. Retinyl esters can also be synthesized by LRAT (32, 35–38). LRAT has a significantly lower K_m for retinol than does ARAT (~2 μM vs. ~15–30 μM), suggesting that LRAT plays the major role in retinol storage when cellular retinol concentrations are relatively low. Indeed, mice lacking LRAT have severe reductions in retinyl ester content in liver, blood, and eyes and exhibit abnormalities of the retinal pigmented epithelium (39). The preferred substrate for LRAT is believed to be retinol bound to cytosolic retinol binding protein, whereas ARAT prefers unbound retinol as a substrate. Therefore, ARAT may play a role in retinol metabolism in tissues with low levels of cytosolic retinol binding protein or when cellular retinol concentrations are relatively high (40), thereby protecting cells from toxicity resulting from excess unesterified retinol. Our data agree with this possible function for ARAT, because hepatic levels of unesterified retinol were increased in *Dgat1*$^{-/-}$ mice challenged with high-retinol diets. The high levels of unesterified retinol in the livers of *Dgat1*$^{-/-}$ mice also raise the possibility that alterations in retinoid metabolism may contribute to the phenotype of mice lacking DGAT1. Retinol is the precursor for retinoic acids (all-*trans* and its isomer 9-*cis* retinoic acids), which are ligands for the nuclear hormone receptors retinoic acid receptor and retinoid-X receptor (41–43), and retinoids affect sebaceous gland function (44, 45), hair growth (46), and proliferation of mammary gland epithelial cells (47, 48), all of which are part of phenotypic changes observed in *Dgat1*$^{-/-}$ mice (17, 22, 23).

Consistent with LRAT being the major enzyme responsible for maintaining retinyl ester levels in mouse liver (39), hepatic retinyl ester content was not reduced in *Dgat1*$^{-/-}$ livers. In fact, the hepatic retinyl ester content in *Dgat1*$^{-/-}$ mice on a high-fat diet was, paradoxically, in-

creased. The explanation for this finding is unclear at present. It is possible that the increases in retinyl ester content resulted from increased LRAT activity or decreased hydrolysis of retinyl esters.

In summary, although both DGAT1 and DGAT2 share diacylglycerol as a common fatty acyl acceptor, the two enzymes differ in their ability to use other acyl acceptors. DGAT1, much more than DGAT2, can catalyze esterification reactions utilizing a variety of fatty acyl acceptors and is, therefore, a less-discriminating enzyme than DGAT2. Our findings indicate that DGAT1 is a multifunctional acyltransferase, and these additional activities of DGAT1 may be relevant to its in vivo functions.

The authors thank S. Cases for providing MEF cells, T. Neidlinger (USDA) for measuring retinoids, and G. Gildengorin (USDA) for statistical analysis of these measurements; S. Erickson, Y. Huang, R. Mahley, K. Weisgraber, and M. Zeiger for comments on the manuscript; S. Ordway and G. Howard for editorial assistance; and B. Taylor for manuscript preparation. This work was supported by National Institutes of Health Grant DK-56084 (R.V.F.), an American Heart Association fellowship (C-L.E.Y.), and the J. David Gladstone Institutes.

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